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Babbs, Charles F.; Pham, Jo Ann; and Coolbaugh, Ronald C., "Lethal Hydroxyl Radical Production in Paraquat-treated Plants" (1989). *Weldon School of Biomedical Engineering Faculty Publications*. Paper 74.  
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# Lethal Hydroxyl Radical Production in Paraquat-treated Plants

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[Plant Physiol. (1989) 90, 1267-1270]

## ABSTRACT

Bipyridinium herbicides, including paraquat and diquat, are believed to act by generating highly reactive, oxygen-centered free radicals within chloroplasts when treated plants are exposed to sunlight. This hypothesis has not yet been confirmed by direct chemical measurements of specific free radicals. We studied paraquat-treated plants using a new method able to detect and quantify formation of highly reactive and deleterious hydroxyl radicals ( $\text{HO}^*$ ), in which dimethyl sulfoxide (DMSO) is used as a molecular probe. DMSO is oxidized by  $\text{HO}^*$  to form the stable, nonradical compound, methane sulfinic acid, which can be easily extracted from plant tissue and measured spectrophotometrically. Initial experiments revealed formation of extraordinary numbers of hydroxyl radicals in light-exposed, paraquat + DMSO treated plants, equivalent at least to the cumulative number of  $\text{HO}^*$  radicals per gram of fresh tissue that would be produced by 10,000 rads of gamma irradiation. This appears to be the greatest production of hydroxyl radicals yet observed in a biological system and is quite sufficient to explain the rapid death of top growth in paraquat-treated plants.

Abbreviations: SOD, superoxide dismutase; MSA, methane sulfinic acid.

Supported by grant by a Focused Giving Grant from Johnson & Johnson, and by grant HL-35996 from the National Heart, Lung, and Blood Institute, U.S. Public Health Service.

## INTRODUCTION

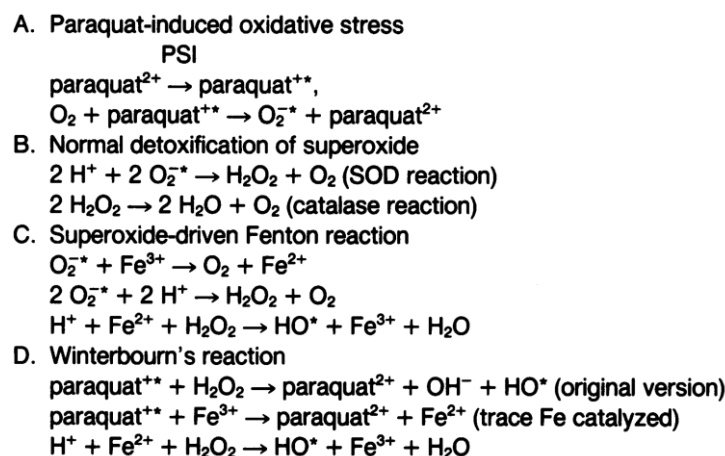
Bipyridinium herbicides are widely used in agriculture because they are inexpensive and highly effective against a wide variety of weeds. Since these herbicides are quickly rendered inert by absorption to clays in soil and also by bacterial action, weeds can be sprayed and useful crops planted with minimal tilling of the soil and minimal soil erosion. The currently accepted mechanism of action of bipyridinium herbicides, for which paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride) is the prototypic compound, involves light-catalyzed generation of oxygen containing free radicals within chloroplasts (11, 16). Free radicals are highly reactive chemical species having an unpaired valence electron, herein denoted as \*. Of late, oxygen-centered free radicals, including the superoxide anion,  $O_2^-*$ , and the highly reactive hydroxyl radical,  $HO^*$ , have been proposed as a new class of pathogens in biology and medicine (3, 8, 13, 15, 21, 24) that can be placed in the sequence: parasites, bacteria, viruses, auto-antibodies, free radicals--in order of decreasing physical size and roughly increasing difficulty of detection.

Hydroxyl radicals, in particular, are among the most highly reactive molecular species known. They are generally considered to initiate a major fraction of radiation-induced damage to living tissues when high energy  $\gamma$  rays split the hydrogen-oxygen bond in intracellular water:  $HOH \rightarrow H^* + ^*OH$  (25). In the presence of suitable chelated transitional metals, particularly iron, hydroxyl radicals can also be formed from superoxide and hydrogen peroxide at ambient temperatures and neutral pH by the iron-catalyzed, superoxide-driven Fenton reaction (Table 1C) (2, 13, 14, 24).

According to the free radical hypothesis explaining the effect of paraquat upon green plants (12, 16, 27), the divalent paraquat cation,  $paraquat^{2+}$ , accepts an electron from PSI in chloroplasts to produce the blue, monocationic paraquat radical,  $paraquat^{+*}$ , which in turn is rapidly oxidized by molecular oxygen to regenerate  $paraquat^{2+}$  with concomitant reduction of oxygen to superoxide,  $O_2^-*$  (Table 1A). The paraquat molecule is then ready for another cycle of reduction and oxidation. A portion of the excess superoxide, either spontaneously or in the presence of SOD, is converted to hydrogen peroxide. Superoxide and hydrogen peroxide that escape detoxification (Table 1B) by superoxide dismutases, catalase (in peroxisomes), and peroxidases may then participate in two pathological pathways leading to the generation of highly toxic  $HO^*$  radicals. The first is the superoxide-driven Fenton reaction (Table 1C), involving trace amounts of iron. The second is a Fenton-like reaction, proposed by Winterbourn (Table 1D) (30), in which the reduced paraquat radical,  $paraquat^{+*}$ , reacts with hydrogen peroxide either directly (30) or in the presence of very small, catalytic concentrations of iron (C. Winterbourn, personal communication, February 1989).

Having developed a new method to trap and quantify  $HO^*$  radicals in living systems (4, 5), we conducted experiments to measure  $HO^*$  generation in paraquat-treated leaves and thus confirm or deny free radical hypotheses regarding paraquat's effect. We used DMSO as a molecular probe for  $HO^*$  and assayed MSA, the immediate nonradical product of the trapping reaction:  $CH_3-SO-CH_3 + HO^* \rightarrow CH_3SOOH + ^*CH_3$ , in which DMSO reacts with a hydroxyl radical to yield methane sulfinic acid and a methyl radical (10, 17, 18). In the presence of DMSO, MSA accumulation acts as a marker for  $HO^*$  generation.

**Table 1. Model of Paraquat-Induced Free Radical Generation**



Because DMSO is exceedingly nontoxic and can be tolerated by living systems in up to 1 M concentrations (1, 7, 19, 20, 28, 29); because it is rapidly absorbed and distributes to all tissue compartments (9); and because it yields a single, stable product (MSA) upon oxidation by  $\text{HO}^{\cdot}$ ; DMSO is a nearly ideal molecular probe for trapping  $\text{HO}^{\cdot}$  radicals. A further advantage in using DMSO to trap hydroxyl radicals is that one can estimate actual numbers of  $\text{HO}^{\cdot}$  radicals produced--as opposed to simply detecting the presence of  $\text{HO}^{\cdot}$  radicals without being able to specify how many were generated.

Hydroxyl radicals are so reactive that they readily combine with normal cellular constituents at frequencies within one or two orders of magnitude of their collision frequency (23). Unless the molecular probe is present in high concentrations, all but a tiny percentage of  $\text{HO}^{\cdot}$  radicals will react with endogenous biomolecules, rather than with the introduced molecular probe. One therefore has no way of knowing what fraction of them was actually trapped, or in turn the total number of  $\text{HO}^{\cdot}$  radicals generated. Thus, to quantify the numbers of  $\text{HO}^{\cdot}$  radicals generated in living tissue, the concentration of the trapping agent must be relatively high, in practice about 0.1 M or greater for DMSO (6, 22); so that the probability that an  $\text{HO}^{\cdot}$  will react with the trapping agent, rather than a host biomolecule is large. In the present studies, we used 5% (0.7 M) DMSO as a molecular probe for  $\text{HO}^{\cdot}$  generation in paraquat-treated plants.

## **MATERIALS AND METHODS**

### **Treatment of Plant Material**

To estimate  $\text{HO}^{\cdot}$  generation in vivo, we measured MSA produced in the small aquatic plant, duckweed (*Lemna minor*), and in perennial ryegrass (*Lolium perenne L.*) treated with DMSO and paraquat, compared to controls treated with paraquat only, DMSO only, or neither agent. Flats of

ryegrass were exposed to natural sunlight (about  $2000 \mu\text{E m}^{-2} \text{s}^{-1}$ ) for 1 to 4 d during July to August, until paraquat-treated leaves became brown. In similar experiments, cultures of duckweed were exposed to fluorescent light ( $830 \mu\text{E m}^{-2} \text{s}^{-1}$ ) for 24 h in an environmental control growth chamber at 20 to 22 °C. In the studies of ryegrass, DMSO was applied as a 5% solution in water, so that it thoroughly soaked the sod. Paraquat (0.1% of the commercial concentrate, Gramoxone, from ICI Americas) was applied once as a mist spray until dripping to the foliage prior to light exposure. In studies of duckweed the small aquatic plants were suspended in 5% DMSO and 0.1% paraquat was sprayed liberally over exposed surface leaves. After light exposure, 2 to 5 g of leaves were harvested for analysis of MSA content by a modification of a colorimetric assay previously described (4) after quick-freezing with liquid  $\text{N}_2$ , grinding with a mortar and pestle, aqueous extraction of the resulting powder with 15 to 20 mL distilled water, centrifugation to remove solid materials, and concentration of the aqueous supernatant by lyophilization. The sulfinic acid assay included two stages: removal of interference with the aid of Sep-Pak columns, and a color reaction with fast blue BB dye.

### Removal of Interference with Sep-Pak Columns

**Principle.** Many potentially interfering species are anionic detergents, such as free fatty acids, which form salt-like complexes with the fast blue BB dye used for the color reaction in the subsequent colorimetric assay (5). Most interfering anions are much less hydrophilic than MSA itself. To remove a portion of such interfering species, a preliminary extraction of the sample with toluene/n-butanol (3/1) at neutral pH may be used. Further removal of detergent-like interference can be obtained by adding fast blue BB dye to the sample and applying the mixture to a lipophilic (C18 Sep-Pak) column. When the column is subsequently eluted with water, the complexes of dye and lipophilic anions remain on the column, while sulfinic acid is readily eluted and collected. In this way detergent-like anions that are specifically capable of complexing with fast blue BB dye to form lipophilic salts are sequestered on the column. The color reaction with additional fast blue BB dye and toluene/n-butanol extraction is then run on the effluent fraction containing the sulfinic acid.

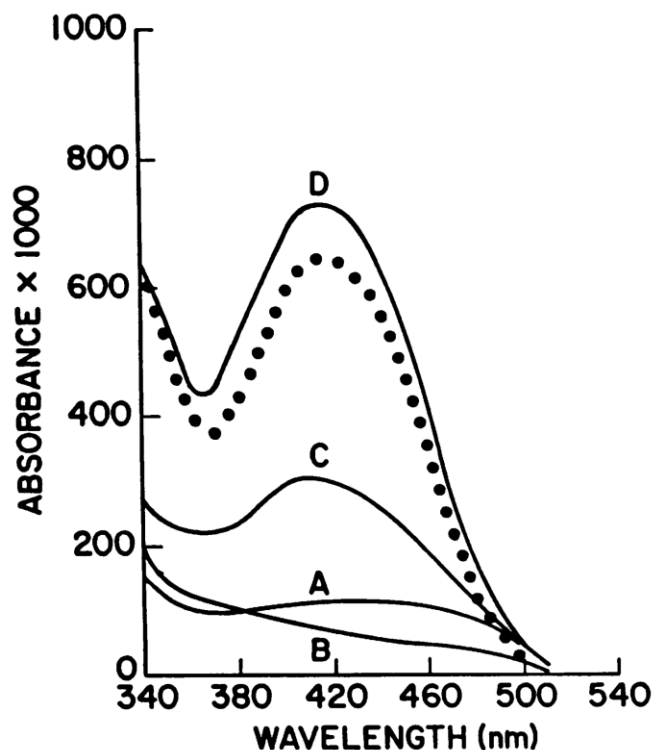
**Procedure.** Lyophilized samples were resuspended in 1 mL of distilled water and extracted twice with 2 mL of toluene/n-butanol (3/ 1). The pH of the aqueous phase was adjusted to 2.5 by addition of HCl. Then 100  $\mu\text{L}$  of 30 mM fast blue BB salt was added to the aqueous sample with mixing, which was applied to a Sep-Pak C18 column (Cat. No. 51910, Waters Associates, Milford, MA 01757) that had been pre-eluted with 2 mL methanol and 2 mL water. The methane sulfinic anion was then eluted from the column with distilled water. The first 1.3 mL of effluent was discarded, and the next 1.5 mL collected for assay by the color reaction.

**Color Reaction.** The 1.5 mL aqueous volume collected from the column was transferred to a test tube, the pH was adjusted to 2.5 by the addition of HCl, and the color reaction was begun by and kept in the dark). Ten minutes were allowed for product development at room temperature in the dark. Then 1.5 mL of toluene/n-butanol (3/1) was added and mixed thoroughly with the aqueous phase for 60 s on a Vortex mixer to extract the yellow, hydrophobic reaction product. After low speed centrifugation to separate the phases, the lower phase, containing unreacted diazonium salt, was removed by aspiration and discarded. The toluene/n-butanol phase was washed with 2

mL of n-butanol-saturated water for 30 s to remove remaining unreacted diazonium salt. The tubes were centrifuged at 500g for 3 min, and the upper phase, containing the diazosulfones, was transferred to a cuvet. One-tenth mL of pyridine/glacial acetic acid (95/5) was added to stabilize the color, which otherwise fades gradually at acid pH. The absorbance as a function of the wavelength from 340 to 520 nm was recorded on a stripchart recorder, using a blank prepared beginning with 1.5 mL of distilled water carried through the same procedure. The sulfinic acid content was then calculated from the absorbance at  $\lambda_{\text{max}} = 425$  nm, with reference to a standard curve.

## RESULTS AND DISCUSSION

The results of a typical experiment using *Lemna minor* are presented in Figure 1 as visible absorbance spectra from the sulfinic acid assay of whole plants exposed to the various treatments. Standard, authentic MSA produces a characteristic absorbance peak at 420 nm (dotted curve). Plants treated with both paraquat and DMSO (D) produced a large, similarly shaped peak. The sharp peak for DMSO + paraquat-treated leaves was characteristic of all experiments. The smaller peak for paraquat-only-treated leaves (C) was common for duckweed but not for ryegrass. Leaves treated with DMSO, without paraquat (B) or either agent (A) generated no significant signal at 420 nm.



**Figure 1.** Data from typical experiment with duckweed (*Lemna minor*), in which hydroxyl radicals were trapped with DMSO and measured as methane sulfinic acid. Visible absorbance spectra of colorimetric assay of untreated leaves (A), DMSO-only-treated leaves (B), paraquat-only-treated leaves (C), and paraquat + DMSO-treated leaves (D). Dotted line represents 300 nmol MSA standard.

Quantitative yields of MSA per gram (fresh weight) of leaves, shown in Table 2, were on the order of 100 nmol/g or 0.1 mM for ryegrass and 475 nmol/g or about 0.5 mM for duckweed. Although this measured production of MSA would represent oxidation of only about 0.01 percent of the DMSO present in the leaves, the cumulative effect of 0.1 mM hydroxyl radicals would be highly toxic to any living system.

**Table 2. Methane Sulfinic Acid Production in DMSO + Paraquat Treated Plants  
Results of Preliminary Studies**

*Quantitative yields of HO\* trapped as MSA per g (fresh weight) of leaves calculated from the absorbance at 425 nm. To correct for false positive, background signal, the mean absorbance per gram for all ryegrass control groups, which did not differ significantly, was subtracted from the absorbance of experimental (paraquat + DMSO) treated ryegrass. Similarly, the absorbance for duckweed controls concomitantly treated with paraquat only, which was the largest control value, was subtracted from that of experimental (paraquat + DMSO) duckweed. Experimental and control plants were grown side by side under identical conditions. Mean control absorbance was about 20% of that for DMSO + paraquat treated plants. The generation of MSA was then calculated from the experimental-control absorbance difference with reference to a standard curve. Results expressed as MSA per gram fresh weight.*

Experiment Number	MSA in Ryegrass	MSA in Duckweed
	<i>nmol/g fresh leaves</i>	
1	67	983
2	27	109
3	124	394
4	149	414

A revealing comparison can be made by calculating the dose of  $\gamma$  irradiation required to produce the same number of hydroxyl radicals as were generated biologically in the paraquat-treated leaves. This can be done by applying the physical formula for the cumulative number of hydroxyl radicals formed per unit volume of  $\gamma$  irradiated water

$$\text{Number of HO}^* \text{ radicals} = \text{radiation dose} \times G_{\text{HO}^*},$$

where  $G_{\text{HO}^*}$  is the radiochemical yield of hydroxyl radicals, a known constant equal to 2.9 molecules per 100 electron volts of absorbed energy (25). After converting units (using  $1 \text{ eV} = 1.602 \times 10^{-19} \text{ J}$  and  $1 \text{ rad} = 0.01 \text{ J/kg}$  absorbed energy), one obtains the expression for the net yield,  $y$ , of hydroxyl radicals in water irradiated by dose,  $D$ , of high energy  $\gamma$  rays as

y (pmol/g) = 3.01 D (rads).

Thus, a yield of 100 nanomoles of hydroxyl radicals per gram = 100,000 pmol/g is equivalent to that caused by a radiation dose of approximately 33,000 rads.

For comparison, a dose of  $\gamma$  irradiation sufficient to kill 97% of cells grown in tissue culture is on the order of 10,000 rads (26). Thus, the cumulative numbers of hydroxyl radicals that we have detected in plants treated with normal, herbicidal concentrations of paraquat are very great indeed and are likely to represent a lethal event. The results provide, we believe for the first time, a direct chemical test of the role of a specific oxygen-centered free radical (HO\*) in paraquat-induced leaf kill, as well as the first direct, chemical demonstration of lethal numbers of oxygen radicals in a biological system that has not been exposed to ionizing radiation.

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